Identification of bacterial microflora in the midgut of the larvae and adult of wild caught *Anopheles stephensi*: A step toward finding suitable paratransgenesis candidates

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\textbf{A B S T R A C T}

To describe the midgut microbial diversity and to find the candidate bacteria for the genetic manipulation for the generation of paratransgenic Anopheline mosquitoes refractory to transmission of malaria, the microbiota of wild larvae and adult *Anopheles stephensi* mosquito midgut from southern Iran was studied using a conventional cell-free culture technique and analysis of a 16S ribosomal RNA (rRNA) gene sequence library. Forty species in 12 genera including seven Gram-negative *Myriodes*, *Chryseobacterium*, *Aeromonas*, *Pseudomonas*, *Klebsiella*, *Enterobacter* and *Shewanella* and five Gram-positive *Exiguobacterium*, *Enterococcus*, *Kocuria*, *Microbacterium* and *Rhodococcus* bacteria were identified in the microbiota of the larvae midgut. Analysis of the adult midgut microbiota revealed presence of 25 Gram-negative species in five genera including *Pseudomonas*, *Alcaligenes*, *Bordetella*, *Myriodes* and *Aeromonas*. *Pseudomonas* and *Exiguobacterium* with a frequency of 51% and 14% at the larval stage and *Pseudomonas* and *Aeromonas* with a frequency of 54% and 20% at the adult stage were the most common midgut symbionts. *Pseudomonas*, *Aeromonas* and *Myriodes* genera have been isolated from both larvae and adult stages indicating possible trans-stadial transmission from larva to adult stage. Fast growth in cheap media, Gram negative, and being dominantly found in both larvae and adult stages, and presence in other malaria vectors makes *Pseudomonas* as a proper candidate for paratransgenesis of *An. stephensi* and other vectors.

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1. Introduction

Malaria is the most important vector-borne disease causing 1–2.5 million deaths annually in many parts of the world (Breiman et al., 2001; WHO, 2010). In spite of employing several control measures, due to emerging insecticide resistance in *Anopheles* mosquitoes, drug resistance in *Plasmodium* parasites, and unavailability of an effective vaccine, malaria still remains frequent and kills millions annually (Hill et al., 2005; Coutinho-Abreu et al., 2009).

Using symbiotic bacteria to deliver the anti-parasitic effector molecules to wild vector populations has been defined as “paratransgenesis” (Beard et al., 2002). In this approach suitable symbiotic bacteria are genetically modified to produce an anti-parasitic factor and then reintroduced into the insect gut, where they inhibit the development or kill the malaria parasites in order to block the transmitted pathogens’ cycle within the vector, or reduce their vectorial capacity (Favia et al., 2007; Aksoy et al., 2008; Coutinho-Abreu et al., 2009).

Generally, symbiotic relationships may award a range of benefits to the host or to the microsymbiont which may be mutualistic or commensalistic. Various phases of their life histories or changing the environmental conditions can change the relationship and its beneficial and detrimental effects to each partner (Klepzig et al., 2009). The symbiont bacteria have been used here for the entire specimen harbored aerobic, facultative that live in symbiosis within the midguts of the mosquitoes. Several characteristics of bacterial symbionts are essential to select proper candidates for paratransgenesis (Beard et al., 2002). One of these important characteristics in selection of potential candidates for paratransgenesis is trans-stadial transmission of bacteria which means the genetically engineered bacteria could be introduced into the larval habitats for colonization in the larval midgut, and then they could be transferred to the adult midgut where...
they can inhibit the development or kill the invading malaria parasites.

The normal midgut microbiota of mosquitoes is still poorly identified and only a few studies have been carried out on microflora of wild caught malaria vectors (Pumpuni et al., 1996; Gonzalez-Ceron et al., 2003; Lindh et al., 2005; Favia et al., 2007; Damiani et al., 2008; Terenius et al., 2008; Rani et al., 2009; Wang et al., 2011). This study was conducted to identify bacterial candidates for a paratransgenic mosquito, via screening cultivable midgut bacteria from larval and adult wild-caught *Anopheles stephensi* mosquitoes, the main malaria vector in Iran and Indian subcontinent (Manouchehri et al., 1976; Sharma, 1995; Oshaghi et al., 2006a; Vatandoost et al., 2006), using 16S rRNA sequences analysis.

2. Materials and methods

2.1. Field collection of *An. stephensi* and isolation of midgut bacteria

Larvae and adult *An. stephensi* specimens were collected from (1) Bandar-Abbas (27.184411, 56.254978) district, an urban and coastal region close to the Persian Gulf and (2) Bashagard district (26.56191, 58.469431), semi mountainous or hilly rural region with many permanent and temporal rivers; both areas are the most important urban and rural malaria foci in southern Iran (Fig. 1).

Larvae were collected from larval habitats using the standard dipping technique (350 ml dipper) and adults were captured from pit shelters and human dwellings by hand catch (Service and Townson, 2002). The specimens were transferred alive to the laboratory of Bandar-Abbas National Institute of Health Research (NIHR). They were identified to species level using standard morphological key (Azari–Hamidian and Harbach, 2009). Adult females and fourth instar larvae of *An. stephensi* were selected for midgut microbiota analysis.

Before dissecting the specimens, the surface of specimens were sterilized with 70% ethanol (Pidiyar et al., 2004) in a sterile hood. Under sterile conditions, specimens were dissected individually and the midguts were mashed and suspended in 500 μL of Brain Heart Infusion (BHI). A 100 μL aliquot of the contents was serially diluted up to 10⁻⁶ and plated onto the following four media of (1) Brain Heart agar (BHA), (2) Nutrient agar (NA), (3) Yeast Extract agar (YEA) and (4) Blood agar (BA) (Mercik, Germany) and incubated at 28 ± 2 °C for 24–48 h. The sterility of all reagents was checked during the entire procedure. Controls for the efficiency of sterilization were treated like other samples. To isolate single purified colonies of the bacteria, continuous sub-culture of every grown bacterial colony was performed. The purified single colonies of the bacteria were later used for DNA extraction and PCR amplification of the 16S rRNA gene.

2.2. 16S rRNA gene amplification and sequencing

Respectively, 45 and 25 purified colonies isolated from larval and adult specimens were selected for molecular identification. Each purified bacterial colony was subjected to genomic DNA extraction using QIALEN DNeasy Kit (Qiagen, Germany) according to the manufacturer instructions. The 16S rRNA universal primers of 16SUF 5’-GAGTTGATCCTGCTCAAG-3’ and 16SR 5’-GTTACCTTGGTGACCT-3’ (Weisburg et al., 1991) were used to amplify about 1.5 kilo base (kb) partial sequence of the 16S rRNA gene from all of the DNA specimens extracted from individual bacteria colonies. The PCR conditions were set as an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56.5 °C for 40 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 8 min.

All successfully amplified 16S rRNA amplicons were directly sequenced by SeqLab (Germany). The probable chimeric sequences were checked with Mallard program (http://www.bioinformatics-toolkit.org) for all acquired sequences and the specimens with suspicious sequences removed from data. Confident sequences were analyzed at the Ribosomal Database Project (RDP II; Michigan State University, http://rdp.cme.msu.edu) and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The similarity of sequences and seq-match analysis were performed using the similarity calculator at the RDP II and NCBI sites. Also ClustalW alignment program was used to check the similarity and distance of sequences (http://www.ebi.ac.uk/clustalw). The BioEdit and MEGAS softwares were used for phylogenetic analysis and tree construction. Position verification were done using distance (neighbor joining) and parsimony (1000 bootstrap replicates) analyses. The sequences were deposited in GenBank with accession numbers (Table 1).

Identification of isolates and their classification in genus and species level was based on sequence comparison with the GenBank and RDPII entries. All specimens with 98% or higher sequence identity with the Genbank entries were assumed for species identification. Also all isolates were identified using classical phenotyping and biochemical methods (Staley et al., 2001) and molecular sequencing results were compared and confirmed results were presented.

3. Results

In this study, midguts of a total of 50 (25/25: urban/rural areas) larvae and 48 (24/24: urban/rural areas) adult *An. stephensi* specimens were dissected and screened for cell free cultivable symbiont bacteria using the culture media explained in Materials and Methods section. Different culture media (BHA, BA, NA, and YEA) revealed different competence for the bacteria growth. BHA provided better growth condition for most of the bacteria symbionts, indicating some species can grow on certain substrates and cannot grow or, perhaps weak growth in other media. According to the 16S rRNA gene sequence similarity rates between the amplified specimens and available data in GenBank and RDPII, more than 28 (62%) cultivable species
<table>
<thead>
<tr>
<th>Bacteria genus</th>
<th>Source</th>
<th>Gram</th>
<th>Accession numbers (AN°) and media used</th>
<th>Closest relative according to BLASTn (accession no., % identity) corresponding to the AN°</th>
<th>No. of purified species/isolates</th>
<th>Isolation area Bandar-Abbas (Coastal) or Bashagard (Hilly)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas</td>
<td>Larvae</td>
<td>–</td>
<td>BH agar: HQ591422, HQ591428, HQ591430, HQ591434-7, HQ613833 Nutrient agar: HQ32857-60, HQ32866-7 Yeast extract agar: HQ32847-51</td>
<td>(FN600406, 96%), (AF364098, 97%), (EU03301, 98%), (HQ018740, 98%), (AE165062, 97%), (EU013945, 97%), (HQ613835, 93%), (FN600408, 98%), (EU013945, 99%), (EU099375, 99%), (AY339889, 98%), (HQ841017, 98%), (AE165063, 99%), (HQ841048, 99%), (CQ281048, 99%), (EU099375, 99%), (AY63221, 99%), (EU013945, 99%), (GU62544, 97%)</td>
<td>34</td>
<td>Coastal and Hilly</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>–</td>
<td>BH agar: HQ613831-35 Nutrient agar: HQ328276 Blood agar: HQ591430, HQ32878-80 Yeast extract: HQ32852-56</td>
<td>(FM213379, 98%), (FN600406, 99%), (FN600408, 99%), (EF426443, 97%), (EF442067, 98%), (CQ89139, 98%), (GU566307, 98%), (EF426443, 99%), (CQ281048, 99%), (EU013945, 98%), (AY633221, 99%), (CQ281048, 99%), (EU099375, 99%)</td>
<td>97%</td>
<td>Coastal and Hilly</td>
</tr>
<tr>
<td>Aeromonas</td>
<td>Larvae</td>
<td>–</td>
<td>BH agar: HQ5914232 Nutrient agar: HQ328262, HQ32864 Nutrient agar: HQ32877, HQ32881</td>
<td>(FJ940838, 98%), (FJ940850, 98%), (FJ461353, 98%)</td>
<td>8</td>
<td>Coastal and Hilly</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>–</td>
<td>BH agar: HQ5914245, HQ541156, HQ591427, HQ591438-40 Nutrient agar: HQ32872</td>
<td>(FJ808727, 99%), (HQ663918, 96%), (GU566308, 98%), (FJ808727, 99%), (GU653918, 97%)</td>
<td>97%</td>
<td>Coastal and Hilly</td>
</tr>
<tr>
<td>Myroides</td>
<td>Larvae</td>
<td>–</td>
<td>Nutrient agar: HQ832865 Nutrient agar: HQ32868, HQ832872</td>
<td>(GU186112, 99%)</td>
<td>3</td>
<td>Coastal and Hilly</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>–</td>
<td>Nutrient agar: HQ83286, HQ32872</td>
<td>(EU331413, 97%), (EU660317, 98%)</td>
<td>97%</td>
<td>Coastal and Hilly</td>
</tr>
<tr>
<td>Exiguobacterium</td>
<td>Larvae</td>
<td>–</td>
<td>BH agar: HQ591425, HQ541156, HQ591427, HQ591438-40 Nutrient agar: HQ32872</td>
<td>(CQ284483, 97%), (EU282457, 89%), (DQ019167, 97%), (EF10829, 93%), (DQ407714, 97%), (GU77059, 97%)</td>
<td>6</td>
<td>Coastal and Hilly</td>
</tr>
<tr>
<td>Shewanella</td>
<td>Larvae</td>
<td>–</td>
<td>BH agar: HQ591421</td>
<td>(FJ689032, 98%)</td>
<td>1</td>
<td>Coastal</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>Larvae</td>
<td>–</td>
<td>BH agar: HQ591441</td>
<td>(FJ514018, 95%)</td>
<td>1</td>
<td>Hilly</td>
</tr>
<tr>
<td>Kocuria</td>
<td>Larvae</td>
<td>+</td>
<td>BH agar: HQ591424 Nutrient agar: HQ32861</td>
<td>(HM854258, 96%), (DQ448711, 99%)</td>
<td>2</td>
<td>Coastal</td>
</tr>
<tr>
<td>Microbacterium</td>
<td>Larvae</td>
<td>+</td>
<td>BH agar: HQ591426, HQ591431</td>
<td>(EU714477, 95%), (HM063035, 97%)</td>
<td>2</td>
<td>Coastal</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>Larvae</td>
<td>–</td>
<td>BH agar: HQ591433, HQ591442</td>
<td>(AB558500, 97%), (AB558499, 99%)</td>
<td>2</td>
<td>Coastal</td>
</tr>
<tr>
<td>Chryseobacterium</td>
<td>Larvae</td>
<td>–</td>
<td>BH agar: HQ591432</td>
<td>(AY468475, 95%)</td>
<td>1</td>
<td>Hilly</td>
</tr>
<tr>
<td>Rhodococcus</td>
<td>Larvae</td>
<td>+</td>
<td>BH agar: HQ591429</td>
<td>(FJ611931, 91%)</td>
<td>1</td>
<td>Hilly</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>Larvae</td>
<td>–</td>
<td>Nutrient agar: HQ32863</td>
<td>(HQ202859, 98%)</td>
<td>1</td>
<td>Coastal and Hilly</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>Adult</td>
<td>–</td>
<td>Nutrient agar: HQ832873, HQ832875</td>
<td>(FJ931841, 99%), (CQ383898, 98%)</td>
<td>2</td>
<td>Coastal</td>
</tr>
<tr>
<td>Bordetella</td>
<td>Adult</td>
<td>–</td>
<td>Nutrient agar: HQ832874</td>
<td>(HQ840720, 97%)</td>
<td>1</td>
<td>Coastal</td>
</tr>
</tbody>
</table>
Fig. 2. Phylogenetic analysis of the isolated bacteria of *Anopheles stephensi* adult (AD) and larvae (L) midgut inferred from 0.7 to 1.3 kb of 16S rRNA gene. Bootstrap values are provided at each node. The scale of genetic distances is shown by the line below the tree where branch lengths are proportional to distance.

(isolates) belonging to ten genera of *Pseudomonas*, *Shewanella*, *Exiguobacterium*, *Klebsiella*, *Enterococcus*, *Microbacterium*, *Chryseobacterium*, *Rhodococcus*, *Kocuria* and *Aeromonas* grew in BHA agar (Table 1). Around 10 (22%) of the cultivable species belonging to the genera of *Myroides*, *Enterobacter*, *Kocuria*, *Aeromonas* and *Pseudomonas* were isolated from NA media and only 5 (11%) species belonging to *Pseudomonas* genera grew in YEA agar (Table 1).

However, the number of bacteria species in the larvae was almost twice as in the adults. More than 40 bacteria species/strains in 12 genera were isolated from the larvae. Seven out of twelve genera were Gram-negative *Myroides*, *Chryseobacterium*, *Aeromonas*,
Aeromonas, Klebsiella, Enterobacter and Shewanella and the rest were Gram-positive Exiguobacterium, Enterococcus, Kocuria, Microbacterium and Rhodococcus (Table 1). In the midguts of adult An. stephensi, 25 cultivable bacteria species all Gram-negative bacteria belonging to Pseudomonas, Alcaligenes, Bordetella, Myroides and Aeromonas genera were identified (Table 1).

Analysis of colony numbers after restriction and purification procedures using dilution and consecutive subcultures showed that 51% of the colonies from the midgut of An. stephensi larvae were Pseudomonas. The rest of colonies in the larvae in descending order were Exiguobacterium (14%), Aeromonas (6.9%), and Kocuria, Klebsiella, and Microbacterium (4.6%). The genera of Pseudomonas and Aeromonas with frequencies of more than 54% and 20% respectively were the most common symbiotic bacteria at the adult stage.

Bio-geographical analysis of the isolated bacteria from adult midguts showed that three out of five genera (Pseudomonas, Aeromonas and Myroides) were found from both coastal and hilly regions. The genus Alcaligenes was isolated only from the hilly specimens whereas Bordetella was found only in the coastal specimens. At the larval stage, genera of Pseudomonas, Exiguobacterium, Aeromonas, Enterobacter and Myroides were isolated from both coastal and hilly regions but Kocuria, Klebsiella, Shewanella and Microbacterium were isolated only from coastal specimens. On the other hand, two genera of Chryseobacterium and Rhodococcus were found only in specimens collected from hilly regions.

Sequence data of the bacteria which were found in both larval and adult stages were selected for phylogenetic analysis in order to find highly associated and possible transstadial species/strains. The phylogenetic analysis produced distinct monophyletic clades for species of Aeromonas, Myroides and Pseudomonas (Fig. 2). The constructed phylogeny revealed the presence of at least four unambiguous monophyletic groups among different species of the most common bacteria, Pseudomonas, one consisting of the highly associated or identical (99–100% similarity) Pseudomonas species/strains isolated from both larvae (L) and adults (AD) of An. stephensi (Fig. 2).

4. Discussion

In the present study 40 and 25 species/strains of bacteria were identified in the An. stephensi midgut at larval and adult stages respectively. The greater numbers of bacteria species identified in this study might be attributable to the origin of the specimens (i.e. wild) as well as using different culture media. The selective pressure of the laboratory conditions may limit bacteria acquisition at both larvae and adult stages (Favia et al., 2007; Gusmao et al., 2010) resulting in great reduction or nil cultivable bacteria in the mosquito midguts (Riehle and Jacobs-Lorena, 2005). For example in a study on midgut microflora of three laboratory colonies of An. stephensi, An. gambiae, and An. albimanus, only nine cultivable bacteria species were identified (Pumpuni et al., 1996). In contrast in studies examining the bacterial composition of midguts from wild An. gambiae and An. funestus from Mali and Kenya (Straif et al., 1998) as well as An. stephensi from India (Rani et al., 2009) 25 and 24 species respectively were identified. In several studies, only one or two culture media were employed (Pidiyar et al., 2004; Gusmao et al., 2010) which are less than the number of media (four) we used. Application of more culture media in this study resulted in a better picture of microflora of the field-caught An. stephensi specimens.

Although most bacteria are lost during metamorphosis from larva to adult stages (Moll et al., 2001), and only a few bacteria are transmitted from larva to adult (trans-stadial transmission). Comparison of the larvae and adult midgut bacterial composition, and the results of the phylogenetic analysis of the sequence data revealed that some Pseudomonas species/strains are present in both development stages indicating possible trans-stadial transmission. However, it is recommended the trans-stadial transmission of those strains be tested experimentally using a phenotypic marker such as a green fluorescent protein (GFP) as used for Asiae bacterium (Favia et al., 2007). This is very important since paratransgenesis using the bacterial delivery systems for malaria control must target the adult mosquitoes (Lindh et al., 2008). These probable trans-stadial transmitted bacteria all are Gram negative and Gram negative bacteria are generally easily transformable (Wirth et al., 1989) and affect the parasite load in the infected mosquitoes and could dramatically reduce either Plasmodium falciparum or P. vivax parasite prevalence and density within vectors (Pumpuni et al., 1993, 1996; Gonzalez-Ceron et al., 2003). Recently another study showed that a Gram negative bacteria “Enterobacter” interfere with P. falciparum development before its invasion of the midgut epithelium (Cirimotic et al., 2011).

In this study Rhodococcus, a Gram-positive bacterium, was found which is also found in Anopheles arabiensis (Lindh et al., 2005). This bacterium is a symbiont in Rhodinus prolixus vector of chagas and has been transformed and used in the paratransgenesis program for control of this disease (Beard et al., 2002).

Riehle and Jacobs-Lorena (2005) determined characteristics of a good bacterium candidate for paratransgenesis in mosquitoes and it seems that several Pseudomonas bacteria isolated in this study address most of needed characteristics and could be good potential candidates for paratransgenesis.

The number of species found in the larval stage was almost twice as that found in the adult stage. It is in accordance with previous studies indicating significant variation in different developmental stages reflecting the effect of environmental habitation of larvae and adult mosquitoes in aquatic and terrestrial ecosystems respectively (Straif et al., 1998; Riehle and Jacobs-Lorena, 2005). Most of the species found in this study have already been reported as normal habitants of mosquitoes midguts (Gonzalez-Ceron et al., 2003; Pidiyar et al., 2004; Lindh et al., 2005; Terenius et al., 2008; Rani et al., 2009; Gusmao et al., 2010), however, three genera of Kocuria, Myroides and Shewanella at larval stage and three genera of Alcaligenes, Bordetella and Myroides at adult stage are the first report on microflora of Anopheles mosquitoes in the literature.

In this study we identified Pseudomonas spp. as the most frequently isolated bacterium from An. stephensi midgut. However, this is in contrast with the previous studies reporting Serratia sp. in An. stephensi midgut (Rani et al., 2009), Bacillus spp. in An. arabiensis mosquitoes (Lindh et al., 2005), and Enterobacter spp. in An. albimanus as the prominent species (Gonzalez-Ceron et al., 2003). This reflects the possible effect of biogeographical factors on the adaptation of bacteria species in each region.

5. Conclusion

Choosing a bacterium for transformation in order to interfere with malaria parasite or shorten the life span of the malaria vector mosquitoes is not simple and several issues should be resolved. Minding all needed characteristics for suitable candidate bacteria for paratransgenesis, we nominate some Pseudomonas species for paratransgenesis program of An. stephensi, however, the aforementioned issues should be addressed to assess the possible use of the candidate bacteria.

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